

## Short Sequence-Paper

*Cyanidium caldarium* genes encoding subunits A and B of V-ATPaseKarl Ziegler<sup>a</sup>, Günter Hauska<sup>b</sup>, Nathan Nelson<sup>c,\*</sup><sup>a</sup> Fachbereich Biologie, Humboldt Universität zu Berlin, Biochemie der Pflanze, Invaliden Strasse 43, 10115 Berlin, Germany<sup>b</sup> Universität Regensburg, Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universitätstrasse 31, 8400 Regensburg, Germany<sup>c</sup> Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA

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**Abstract**

The genes encoding subunits A and B of V-ATPase in *Cyanidium caldarium* were cloned and sequenced. While the gene encoding subunit A is not interrupted by introns, the gene encoding subunit B contains seven introns ranging from 36 to 60 nucleotides.

**Keywords:** ATPase, V-; Gene; Intron; Bioenergetics; (*C. caldarium*)

**1. Introduction**

Numerous organelles of the vacuolar system of eukaryotic cells are energized by V-ATPases and each organelle has a specific requirement for its internal pH and membrane potential [1–5]. Thus endosomes operate with smaller  $\Delta$ pH than the vacuoles or lysosomes, and therefore their V-ATPase has to be regulated in order to obtain the required protonmotive force for each of the organelles. These special requirements may be fulfilled by secondary transport systems such as chloride carriers and regulation of the V-ATPase activity. The internal pH of organelles of the vacuolar system is also variable and tightly regulated. While yeast vacuoles maintain an internal pH of about 5.5 units, it is assumed that the vacuoles of lemon fruit may have a pH as low as 2 units [6]. Similarly, some brown and red alga maintain very low pH in their vacuolar system. Further evidence that the V-ATPase is regulated in plants is provided by the fact that the vacuolar pH can vary in different tissues of the same plant and in the same cell during the course of development or in response to changing environmental conditions. Crassulacean Acid Metabolism (CAM) plants are the classic example of vacuolar pH regulation. The pH of their leaf vacuoles fluctuate from pH 3 at night to pH 6 in the day [7]. During the maturation of citrus lemon fruit the vacuolar pH of the

juice sac cells declines from 5.5 to about 2. This may suggest that in the mature lemon fruit V-ATPase functions at a thermodynamic equilibrium. In most cases V-ATPase operates below thermodynamic equilibrium and it was proposed that a proton slip is one of the main factors in maintaining smaller  $\Delta$ pH in different organelles of the vacuolar system [8]. We elected to utilize the thermophilic and acidophytic unicellular red alga *Cyanidium caldarium* to investigate the underlying mechanism of maintaining relatively low internal pH in their vacuole. The red alga *Cyanidium caldarium* grows in a medium with a pH of 2.5 to 2.7. We assume that the pH of its vacuoles may be lower than the external medium. If this is the case, cloning and sequencing of V-ATPase subunits may reveal the clue for regulating the internal pH of organelles of the vacuolar system. Here we describe the cloning of two genes encoding V-ATPase subunits, subunit A and subunit B from *Cyanidium caldarium*.

*Cyanidium caldarium* was routinely grown in a 12 l glass flask under constant illumination and aeration at a temperature of about 42°C. The composition of the medium was: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/l; K<sub>2</sub>HPO<sub>4</sub>, 20 mg/l; MgSO<sub>4</sub> 7 H<sub>2</sub>O, 20 mg/l; micronutrient solution for BG-11 medium [23], 1 ml/l; yeast extract, 0.1% (w/v); glucose 0.2% (w/v); pH 2.7. Cells were harvested by centrifugation, washed twice with STN-buffer (330 mM sorbitol, 30 mM Tricine, 3 mM EDTA (pH 7.5)), redissolved in STN-buffer and stored frozen at –20°C.

DNA of *Cyanidium caldarium* was prepared according to a published method of Phenol/SDS that was developed

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1	GGATCCTATCCATACTGTCCAGTTTTCACATCACACTGGATATTTAGGTGGCTTTCGTGG	60
61	AAAGAGACTGAGCAGTGAAGAGTTTGATCAACTCATTGCTGGTTTAGAGGCCAACTGTTT	120
121	CTTGCAACAAGTGTAAATATCATTGATGGAAGTAGGTCAGCGGTGTTTGACGACGCATA	180
181	GGGATTATGTCTGTATGGGATATATAGGTGACCATGATTGCTACTTCATATATTGGAAG	240
241	CCGTCAAAAACTCAAGAAACCAATCTTGCTTTGTATGTGATCCCGTAATGGTAGGTA	300
301	CTGCTATGGATGGAATAATATATAGTCTTAGTTGGGTGTGCATAGGGCGATAATGGGA	360
361	AATTGTATGTTCTCCCAAGTCTCGTTCCTATTATCGAGACCAAGTAATTATGCTTGCCG	420
421	ATATTATAACACCGAATCAGTTCGAGTTGTCCATCCTATCCGAGAAAGCTGTGGGTCCG	480
481	TTACAGAGGCATTCCAAGCTTGTAGATATTTACATGAATATCGAAAAGTACAGAATATTG	540
541	TAGTTACTAGTGGAGAAATACGAAGATATGGATCTTTTGTGACTAGATAAGTAGTGACT	600
601	TTGGTAACCAAAAATATATTACAGACAGTAGAGAAGATACAAGTAGTTTCACGGGAGCTG	660
661	GTGACTTGAGTAGTCTCTTATCTTGGCTGGTATGTTATTTGAAAGGTGATATTGTAG	720
721	CTGCTTGTGAAAGAGCAATGGCAAGTGTTCACCGTACTTTGGTACATACGGCAGCGGTAC	780
781	AATCGCCGCCAACTCGGTGGTGGGAATTGGAATTGATTGGCAGTCAACTTTGGTTGAGGA	840
841	ATCCTCCGTTAGGCATCGTGAAGACTCGTGGCATTACACTACAAGATGACAAACAGTTCGA	900
	M T T V R	
901	GTCAACGGAATGAAAAACGGAATAATTAATAAAGATATCCGGTCTGCTGTTCCGAGAA	960
	V N G M K N G I I K K V S G P V V S A E	
961	AATATGGACGGTGTGCAATGTATGAACCTGTTCTGTAGGAAACGAACAACCTGGTGGGT	1020
	N M D G A A M Y E L V R V G N E Q L V G	
1021	GAAATCATCCGTTTGGAGGTTCCGTTGCGACAATCAAGTGTACGAAGAACTTCAGGA	1080
	E I I R L E G S V A T I Q V Y E E T S G	
1081	TTAACTATAGGTGACCCAGTATTGTGACTGGATCGCCACTATCCGTAGAATTGGGACCA	1140
	L T I G D P V L C T G S P L S V E L G P	
1141	GGCTTGATGGGAAATATTTTCGACGGCATACAAAGACCCTCGAGAAAATTCGGGAAAGA	1200
	G L M G N I F D G I Q R P L E K I A E R	
1201	AGCAACTCTGTGTTTATACCAAGAGGAGTAAACGTTCCGGCTTTAGATAGAAAGAAAGTA	1260
	S N S V F I P R G V N V P A L D R K K V	
1261	TGGGAGTTTAGACCTGCAGACAACCTAAAGTAGGAGACCCGATAACTGCTGGAGATATA	1320
	W E F R P A D N L K V G D P I T A G D I	
1321	TACGGCATCGTTCCTGAAACACCTCTAATAGACCACAAGATTATGTTACCGCCAAACCAA	1380
	Y G I V P E T P L I D H K I M L P P N Q	
1381	ATGGGAAAGATAGTGTCTTACGCCACCTGGAGACTATACCTTGGAAAGTACTGTTTGG	1440
	M G K I V F L A P P G D Y T L E D T V L	
1441	GAAATAGACTTCAATGGACAAAAGAAAAGTTCTCCATGGTACATCAGTGGCCAGTAAAG	1500
	E I D F N G Q K K K F S M V H Q W P V R	
1501	CTTCCTAGACCACTGACAGAAAACCTTCGGGCAGATAAACCACTTTTAAACCGGTCAAAGG	1560
	L P R P V T E K L R A D K P L L T G Q R	
1561	GTATTGGACGCTCTCTCCCTTCGTTACAGGAGGAACCTTGTCTATACCAAGTGTCTTT	1620
	V L D A L F P S V Q G G T C A I P G A F	
1621	GGTTGTGGAAGACAGTTATCTCAAGCACTGAGTAAATTTTCAAACCTCAGATGGAATA	1680
	G C G K T V I S Q A L S K F S N S D G I	
1681	GTTTATGTCGGTTGTGGGAAAGAGGCAATGAAATGGCAGAGTATTGAAGGACTTTCCG	1740
	V Y V G C G E R G N E M A E V L K D F P	
1741	GAACTAACCATGACAGTGGGAGATAGAGAAGAAAGTATCATGAAAAGAACTCTTCTGGTA	1800
	E L T M T V G D R E E S I M K R T L L V	
1801	GCAAACTCTTCAATATGCCTGTGGCTGCTAGAGAAGCTTCCATTTATACCGGAATTACT	1860
	A N T S N M P V A A R E A S I Y T G I T	
1861	GTCTCGGAATATTACAGAGATATGGGTTTAAATATAAGTATGATGGCCGACTCTACTTCC	1920
	V S E . Y Y R D M G L N I S M M A D S T S	
1921	AGATGGGAGAACCTTGAGAGAAATAAGTGGAAAGCTGGCGGAAATGCCGGCAGATAGT	1980
	R W A E A L R E I S G R L A E M P A D S	
1981	GGTTATCCTGCATATTTGGCAGCTAGATTGGCAAGTTTTTATGAGAGAGCTGGTAAGGTA	2040
	G Y P A Y L A A R L A S F Y E R A G K V	
2041	TCTTGTTTAGGCTCCCTAATAGACAAGGTTCTATTACTATTGTGGGAGCTGTATCACC	2100
	S C L G S P N R Q G S I T I V G A V S P	
2101	CCAGGTGGTGACTTTTTCAGATCCTGTACGTCAGCCACCTTGGAAATCGTTCAAGTCTTT	2160
	P G G D F S D P V T S A T L G I V Q V F	
2161	TGGGTTTGGATAAGAACTGGCTCAACGAAAGCATTTTCTTCGGTCAATTGGCTCATC	2220
	W G L D K K L A Q R K H F P S V N W L I	
2221	TCCTATTCCAAGTACATGAAGGCATTGGAGCCGATATACGAAGAACGGTTCCCGGAATTT	2280
	S Y S K Y M K A L E P Y Y E E R F P E F	
2281	CTAAACTATCAACAAAGGCTAGAGAAATCTTCAACAGAGGATGATCTAATGGAATA	2340
	L N Y Q Q K A R E I L Q T E D D L M E I	
2341	GTACAACCTAGTAGGAAGGACTCGCTGGCAGAAAACGATAAGATTACGCTGGAAGTTGCA	2400
	V Q L V G K D S L A E N D K I T L E V A	
2401	AAAATGATACGTGAAGACTTTCTGCGCAAAATTCGTTTACTGAATATGACCGATTCTGT	2460
	K M I R E D F L A Q N S F T E Y D R F C	
2461	CCATTTTACAAGAGCGTTCTCATGTTGCGCAATATGATTCAATTTTATGAGTTGGCAAAT	2520
	P F Y K S V L M L R N M I H F Y E L A N	
2521	AAGGCAGTTGAAGGATCCGGTGAGCAACATCTAACATTGGCGCAGATAAGGAACAAATG	2580
	K A V E G S G E Q H L T L A Q I K E Q M	
2581	GGTGAACCATCTACAAGATATCTGGTATGAAGTTTTTGGATCCTGCACAAGTTGGTCT	2640
	G E T I Y K I S G M K F L D P A Q G W S	
2641	CTTTTGAACATTGCAGTGGGTTTATATTACTGGGTTGTAGGTGAAGATGCTTTGAGA	2700
	L F *	
2701	AGTAAGCTCGATGCACTGTACTCGCAGATAACAGACGGCTTTTACAAAATGGAGAATAGT	2760
	CTATGACATTAAAGGAAACAGTATATAAAGATGTCGCACTGAATATTTAGTTGCGTTGT	2820
2761	TTGTCGAACAGTCTTTGGTATACTTCACAAAAAGCCTTTATCGTAATACAAAACGGCAA	2880
2821	CACCTAAAACGGGTAGAAGCACAGAAGATATCTCAACGAATTGCGCCAAATCATCTCCGT	2940
2881	CCACTTTTTTCAAATTTTTTTCGTCTTTTTTTTGGCGCCAACTTTTGGGATTTTGGC	3000
2941	GCCTTCACTTTTCCGTACAAAATTTTCTATTCTTGTAGGAAAAGGATGACAGAGTGGGA	3060
3001	TGCTCAACCACTGATTACGGTGTCCAACTTTCAGAAATATGAAAGGAATATAAATGA	3120
3061	ATGTCAAGATTTCGACGCAAAAGAAAATTTAGAGAGTCTTCTTGCCTACACTTCCAG	3180
3121	TGAATCATGTTTCAAGTATAGAGAAGCTT	3211

Fig. 1. Nucleotide and deduced amino acid sequences of the gene encoding subunit A of V-ATPase from *Cyanidium caldarium*. GenBank accession number U17100.

1	TCTAGAAAGGAACGACCTTTTCTTTCCACGTGTTTGCTACTGGTTGTTCCAGCATA	60
61	TCAGGATGCAACTGTTTGACAAAGCAAAATATCTTTGCTAAAACAGGCAAAAGAGTTGG	120
121	CACATGGATATACGTGTGAACAGAAACACGATACCTTTACTGTTCAAAAGAACACACA	180
181	GGACTCCAAGTCCAGAACTGAAAAAGGGTCTCTTTCTGACCATGCAACAACTTTTGTAG	240
241	ACAATTATGGAAACAAATATTTACAGGACCAAGTATTGACTTGGAAAGAAATGTTTGCT	300
301	ACTTGCAAAATAGCATTCTTCAGCGAAAGACTGCAGCAACTGTGGAATTTGGTTGGAACG	360
361	AGCGAGCTGCTGCTTGTGGATGTCGCGATTCTTCATCAGATCATCCATAGGAAGAGTAGT	420
421	TTGATTTCTTTATAGATTTTACAATAGTATTGTATACATATCAGGCATGTTTGGATCT	480
481	CTTTTGCATTACACACACAACTCTCTCATATATATATGATATATATGATGTCTGTA	540
541	GATGTATATATATGTAACATCGAATCATTTCAACGAATTTGGCTCTTTCCACATTGTGC	600
601	TCTTGTGCATTTTATGGATTGGGCTTGTGGCGCTTTCTTTGGCTTCTGACAGAG	660
661	CAACGGAAGAAGCTATTCACTTATGGCTAGTCGTAAGTATAGATAATTGAAAAACGGA	720
721	CTACTATTGGTGTCAACAAAGTAAATAGCAGAGTCACAGCTTATAGACCATATGAGGCT	780
	M E A	
781	TTCAACTTGCACAAGtaagaagaacagaagcctcgttgcgcaacaacacacaggg	840
	F N L H K	
841	ttagAAAGCAGTATCAAGAGATTACATTGTGAAGCCTAGACTCGAATATAGAACTGTATC	900
	K A V S R D Y I V K P R L E Y R T V S	
901	AGCTGTAACCGTCTTTAATATCTCTGCAGAACGTCAGTCACCAAGATTTCGCAGAAAT	960
	A V N G P L I I L Q N V K S P R F A E I	
961	AGTAAACGTAACGCTGGGGATGGAAGTGTGAGAAGGGGCCAAGTGTGGAATCAACCA	1020
	V N V T L G D G S V R R R G Q V L E I N Q	
1021	AGACAAAGCTGTGGTGCAGtgagtagtctatttcgaggagtggttcagcttagtcttc	1080
	D K A V V Q	
1081	gtgaagattTTTGAAGGGACAACAGGCATCGATAATAAGAAAACCGTTTGTCAAGTTTAC	1140
	I F E G T T G I D N K K T V C Q F T	
1141	TGGAGAAATATTAACACACGATATCGTTGGATATGTTGGGTAGAGTATTCACCGGTC	1200
	G E I L K T P V S L D M L G R V F N G S	
1201	TGGAAAGCCAATCGACGGTGGTCTCTCTATCTGCTGAAGCATATTAGATATTCAAGT	1260
	G K P I D G G G P P I L P E A Y L D I Q	
1261	aagtagtagcgtgctttcaacaattattgttgaacatacaaatgctcgtatagGGT	1320
	G	
1321	CAGCCTATTAATCTCAGAGTCGGACCTATCCGAAAGAAATGTTGAAACAGGTATATCA	1380
	Q P I N P Q S R T Y P E E M F E T G I S	
1381	AGTATTGACGTGATGAACCTTATTGCAAGAGGTCAAAGATTCCACTCTTTCTGGAGCA	1440
	S I D V M N S I A R G Q K I P L F S G A	
1441	GGTTTACCACATAATGAAGTGGCTGCACAAATATGTAGACAGGTATGCTTGTGCTCACT	1500
	G L P H N E V A A Q I C R Q V C L V S T	
1501	TGCAgtttgtaaactgtctcaattctatgggaataggcagCCCTTGGTAAAGCGTTCGGGT	1560
	C T L V K R S G	
1561	AAAGATGAAGAAGACTTGTCTATAGTGTTCGAGCAATGGGTGTCAATATGGAACCTGCA	1620
	K D E E D F A I V F A A M G V N M E T A	
1621	CGATTCTTCAGACAAGACTTTGAAGAGAATGGTGAATGGAACAGTAACCTTTATCTTG	1680
	R F F R Q D F E E N G A M E R V T L F L	
1681	AATTTGGCAAACGACCCGACTATTGAACGTATTACTCTCGTTGGCACTGACTTTT	1740
	N L A N D P T I E R I I T P R L A L T F	
1741	GCAGAAATCTTGTCTTATGAAAGGGAACATGTATTGGTTATCTTGACAGATATGAGT	1800
	A E Y L A Y E K G K H V L V I L T D M S	
1801	GCCTATGCAGATGCTTTGCGTGAAGTTTCTGCTGCACGTGAAGAGgttgattattttgt	1860
	A Y A D A L R E V S A A R E E	
1861	ggttgggttgcttatatgcttcttattattattgtcctgtagGTACCAGGAAGAAGA	1920
	V P G R R	
1921	GGCTATCTGTTTATATGTACACGGATTGGCAACCATTATGAGAGAGCTGGTCGAGTG	1980
	G Y P G Y M Y T D L A T I Y E R A G R V	
1981	GAAGGACCCCTGGTTCCATTACTCAGTTCGGATATTAACGATGCCAAATGACGATATT	2040
	E G R P G S I T Q L P I L T M P N D D I	
2041	ACGCATCTTATTCGGATCTTACTGGtaataggagaatatttgaatttgcgactaa	2100
	T H P I P D L T G	
2101	cacgtagctctagATATATCACCGAGGAACAAATTTACTGGATCGACAGTTACATAATA	2160
	Y I T E E Q I Y L D R Q L H N R	
2161	GACAGATATATCCCCCATCAATGTGCTTCTTCACTATCTGACTTATGAAGTCCGCTA	2220
	Q I Y P P I N V L P S L S R L M K S A I	
2221	TTGGCGAAGGAATGACCAAGAGGATCATTGGATGTATCCAATCAATTGTATGCTGCTT	2280
	G E G M T R K D H S D V S N Q L Y A A Y	
2281	ATGCTATGGGAAAAGATGCCTTGGCTATGCGTGCAGTAGTTGGTGTGGAAGCTTTGTCCC	2340
	A M G K D A L A M R A V V G V E A L S Q	
2341	AGGAAGACTTGTCTTATTGGAATTTTCATGACAagtaagtgctggactatctcatgccc	2400
	E D L L Y L E F H D K	
2401	tgttttcatgattattgtagGTTTGAGAGCGTTTTGTGAATCAAGGAGCCTATGAAAGA	2460
	F E R R F V N Q G A Y E R	
2461	AGAGATATATATCTCGTTAGATATGCGTGGGATTGTTGCGAATATTTCCGGTGCAG	2520
	R D I Y T S L D M A W D L L R I F P V Q	
2521	ATGTTGCGTCTATTCTGAGAAAATAGTCAAGAAATATTATCATCGGACGAGTAATTAT	2580
	M L R R I P E K I L Q E Y Y H R T S N Y	
2581	GAACACAAGGAGAACAGCCGATTCGAGTGTGTTGTAATTCATTGTTGGTATACCT	2640
	E H K E N K P H S S R S *	
2641	TCATAGTTTCTTGATGTAGTAGTTTCTTATCTAAGAAAGCATAAAAAATGATTTT	2700
	CTAGACTCCGAGAGCTACTAGAAGGCTTTGATCTAGGCCATCAACAGAGTTGTAATA	2760
2761	GTTGTATATGTCGTATTACAACTATAATAAATGGCCCTTGACGCGCTGTACCGACA	2820
	ACAACAACAACAATAAAAAACACAGCACTGGTTGTCCACAGATACTTTATCTATAT	2880
2881	TCTTATGCTATCCAACCTGACTCTTCTTGTGCTGGTTGTGTTTGTCTTCATAGAGAGCA	2940
	GCAACTGACCGACGAAGCTCGAAACGGCCATTAAAGAAATTTGTTTCATCTCTCGCAGT	3000
2941	GCAACACCGCAATATGTTCAACGCTTGGATGGGAGCTGCAGTAGGCTTAGCAGCAAC	3060
	TGCTTACCAACGGTTCAAGACTACTTCCCTTCTCGGCTGAGTTGCCTGTTCTTAGAC	3120
3061	AATCGCTTTGGTTGAAACCTGTTGGTTTCTTAGGTCCTTGGGAACACTTGGCTGCGAT	3180
	GGCACTCGGTGCTTACATCGGCAAAATATGGGAGAAATACCAACATGACAAGATTGAAG	3240
3241	AAAGGAGTATCGTATGAAGAGAGATCTTGAACGTGCAAGAGAGAAAATCAAAATGCGCT	3300
	TGAACAAGTTGCAGCAGAGAACGGAGCTTGGAGGTTGTGTATGTATGAAAACAAGCTC	3360
3301	GTGAACCAACAACCTTGTCTGGGTATATTTATATATTTGATATCAATAATAACACACAA	3420
	CTTGGGTGACTTTTCAAGGCAACAACGATTCACTTCTCTTCTACTATTATTATTATTA	3480
3481	TGTCAACTACACAAATTTGTATACAGATAACTGATTCCTTGTATTTCCCATAAACCACTGT	3540
	TCTCCGACAGAAACATAGCAACCGAATAGTTGACGTTGATTCATTCTTGGTTTGTG	3600
3541	TCCGCGGAGATATGCCAAACACATGAAGTAGGATGCTATCTTGAATCCCCAAGTACTC	3660
	GTACGAGTTTCCAATATGGGACAGAATGAACCATCTCCATGACTTTTCTACTGCTGCAT	3720
3721	GTAGAAGAATCCGTGGTAGCACTCTTGTCTGTGCTCTTGTATATCTAGA	3773

Fig. 2. Nucleotide and deduced amino acid sequences of the gene encoding subunit B of V-ATPase from *Cyanidium caldarium*. GenBank accession number U17101.

for preparation of plant DNA [9]. All the DNA libraries were constructed in the same way: *Cyanidium caldarium* DNA was digested completely with restriction enzyme, fragments of appropriate size were isolated and cloned into the respective restriction site of plasmid pBluescript II SK+. The resulting constructs were used for transformation of competent *E. coli* strain DH5 $\alpha$ , ampicillin resistant cells were recovered. Screening of the libraries was carried out with <sup>32</sup>P-labeled probes by repeated colony screening and subsequently by dot blot analysis [10]. Labeling of the probes was performed by Random priming with Klenow-Polymerase.

For sequencing the cloned fragments, overlapping deletions were generated by the method of Henikoff by unidirectional digestion with exonuclease III [11]. The resulting sets of truncated DNA and the original fragments were sequenced with T<sub>3</sub> and T<sub>7</sub> promoter primers. Second DNA strand was sequenced using internal primers.

#### Screening for the gene of subunit A

A DNA library was constructed from BamHI-DNA fragments of 1.5 to 10 kb clone into BamHI site in pBluescript SK+. The resulting library consisted of about 20 000 independent colonies. Screening for the gene encoding subunit A was performed by using a 0.8 kb cDNA fragment from bovine adrenal medulla together with a 0.65 kb fragment of the corresponding yeast gene. About 70 000 colonies of the *Cyanidium* library were screened and the positive colonies were analyzed by dot blot. Four clones giving strong signals with both probes were obtained. All of them contained the same insert of 2538 bp (nucleotides 1 to 2538 of the sequence). Sequencing showed that the DNA fragment is missing the part of the gene encoding the C terminal part of the protein. A partial DNA library of 1.0 to 1.35 kb HindIII fragments of *Cyanidium* DNA was prepared and screened with a 0.19 kb SpeI/BamHI fragment of the *Cyanidium* gene (nucleotides 2346 to 2538 of the sequence) as a probe. Three positive clones were obtained, all contained the same insert of 1371 nucleotides (nucleotides 1840 to 3211 of the sequence). Sequence analysis of the BamHI and HindIII DNA fragments showed that both overlapped with about 700 bp. The alignment of the sequences resulted in the complete sequence of the *Cyanidium* gene of subunit A.

#### 1.2. Screening for the gene of subunit B

For isolating the gene encoding subunit B, a 1.1 kb bovine cDNA together with a 1.4 kb fragment of the corresponding yeast gene were used as probes [20]. About 120 000 colonies of the above *Cyanidium* library were screened with the above labeled DNA fragments and positive colonies were analyzed by dot blot hybridization. Four clones which gave strong signals with both probes were sequenced and showed high homology to genes encoding subunit B of V-ATPase from other sources. After XbaI

digestion, two fragments of about 1.7 and 2.1 kb were identified by Southern blots analysis. Both fragments were cloned into pBluescript II SK+ and sequenced. The analysis of the sequences showed that each of the 2 XbaI fragments contained a part of the gene and when aligned gave the complete sequence of the gene encoding subunit B.

Fig. 1 shows the nucleotide and deduced amino acid sequences of the *Cyanidium* gene encoding subunit A of V-ATPase. The deduced amino acid sequence contains 587 amino acids and it is highly homologous to other amino acid sequences of subunit A of V-ATPase from other sources. A search in the GenBank revealed that the *Cyanidium* subunit A is 73% identical to the corresponding subunit from bovine [13] and about 70% identical to the corresponding subunits from the insect *Manduca sexta*, the fungi *Neurospora crassa* and *Saccharomyces cerevisiae* or the plant carrot [14–16]. It also exhibited over 50% identity with the corresponding subunit A of the enzymes from *Archaeobacteria* [17,18]. Its high identity with subunits from mammalian sources vs. those of fungal and plant is somewhat surprising. The open reading frame is uninterrupted with exons and it has no unusual features. In contrast to subunit A, the gene encoding subunit B is interrupted by 7 introns (Fig. 2). The size of the intron ranges from 36 nucleotides up to 60 nucleotides. After computer splicing of the introns the reading frame contains 500 amino acids. The deduced protein is highly homologous to subunit B from other sources. It exhibits nearly 80% identity with the corresponding V-ATPase subunits from bovine, plants and the insect *Manduca sexta* [19,20]. The corresponding subunit of *Saccharomyces cerevisiae* and *Neurospora crassa* are only about 72% identical to the *Cyanidium* subunit [12,21]. As subunit A, subunit B also has over 50% identity with the corresponding subunit from archaeobacteria [18,22]. Table 1 depicts the percentage identity of the amino acid sequences of subunits A and B

Table 1  
Sequence homology of the *Cyanidium* subunits A and B with corresponding subunits from various sources

Source	Subunit	% Identity	Accession number	Ref.
Bovine	A	73	X58386	[24]
	B	77	M83131	[19]
Human	A	73	L09235	[25]
	B	76	X62949	[26]
<i>Manduca sexta</i>	A	70	S94847	[27]
	B	77	S43760	[28]
Carrot	A	73	J03769	[16]
<i>A. thaliana</i>	B	80	J04185	[20]
<i>S. cerevisiae</i>	A	70	J05409	[14]
	B	72	J04450	[12]
<i>Entamoeba histolytica</i>	A	70	U04849	[28]
<i>Plasmodium</i>	A	63	L08200	[29]
<i>falciparum</i>	B	69	U03915	[30]
<i>Trypanosoma</i>	A	68	Z25814	–
	B	71	Z25815	–

of *Cyanidium* V-ATPase with corresponding subunits from other sources. The percentage identities suggest closer relation to mammalian subunits than to the fungal ones.

The gene structure encoding subunit A and subunit B is quite interesting. While the gene encoding subunit A has no intervening sequences, the one encoding subunit B contains several of them. Being unaware of other nuclear genes that were cloned and sequenced from *Cyanidium caldarium*, we cannot know whether this is a general pattern of this organism or if it is a unique situation for the V-ATPase subunit. An attempt to identify the source of the two genes evolutionary tracing did not give significant results (Lill and Nelson, unpublished). Further studies on the genes encoding other subunits of V-ATPase from *Cyanidium caldarium* may shed light on the control mechanisms of internal pH in the vacuolar system.

## References

- [1] Mellman, I., Fuchs, R. and Helenius, A. (1986) *Annu. Rev. Biochem.* 55, 663–700.
- [2] Nelson, N. (1989) *J. Bioenerg. Biomembr.* 21, 553–571.
- [3] Nelson, N. (1992) *Biochim. Biophys. Acta* 1100, 109–124.
- [4] Taiz, L. (1992) *J. Exp. Biol.* 172, 113–122.
- [5] Forgac, M. (1989) *Physiol. Rev.* 69, 765–796.
- [6] Sze, H. (1985) *Annu. Rev. Plant Physiol.* 36, 175–208.
- [7] Lutge, U. (1987) *New Phytol.* 106, 593–629.
- [8] Moriyama, Y. and Nelson, N. (1988) in *The Ion Pumps, Structure, Function and Regulation* (Stein, W.D., ed.), pp. 387–394. Alan R. Liss, New York.
- [9] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) *Current Protocols in Molecular Biology*, Vols. I and II, John Wiley & Sons, New York.
- [10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn., Cold Spring Harbor Laboratory, New York.
- [11] Henikoff, S. (1984) *Gene* 28, 351–359.
- [12] Nelson, H., Mandiyan, S. and Nelson, N. (1989) *J. Biol. Chem.* 264, 1775–1778.
- [13] Puopolo, K., Kumamoto, C., Adachi, I. and Forgac, M. (1991) *J. Biol. Chem.* 266, 24564–24572.
- [14] Hirata, R., Ohsumi, Y., Nakano, A., Kawasaki, H., Suzuki, K. and Anraku, Y. (1990) *J. Biol. Chem.* 265, 6726–6733.
- [15] Bowman, E.J., Tenney, K. and Bowman, B.J. (1988) *J. Biol. Chem.* 263, 13994–14001.
- [16] Zimniak, L., Dittrich, P., Gogarten, J.P., Kibak, H. and Taiz, L. (1988) *J. Biol. Chem.* 263, 9102–9112.
- [17] Denda, K., Konishi, J., Oshima, T., Date, T. and Yoshida, M. (1988) *J. Biol. Chem.* 263, 6012–6015.
- [18] Inatomi, K.I., Eya, S., Maeda, M. and Futai, M. (1989) *J. Biol. Chem.* 264, 10954–10959.
- [19] Puopolo, K., Kumamoto, C., Adachi, I., Magner, R. and Forgac, M. (1992) *J. Biol. Chem.* 267, 3696–3706.
- [20] Manolson, M.F., Ouellette, B.F.F., Filion, M. and Poole, R.J. (1988) *J. Biol. Chem.* 263, 17987–17994.
- [21] Bowman, B.J., Allen, R., Wechsler, M.A. and Bowman, E.J. (1988) *J. Biol. Chem.* 263, 14002–14007.
- [22] Denda, K., Konishi, J., Oshima, T., Date, T. and Yoshida, M. (1988) *J. Biol. Chem.* 263, 17251–17254.
- [23] Schlosser, U.G. (1994) *Botan. Acta* 107, 113–186.
- [24] Pan, Y.X., Xu, J., Strasser, J.E., Howell, M. and Dean, G.E. (1991) *FEBS Lett.* 293, 89–92.
- [25] Van Hille, B.J.M., Richener, H., Evans, D.B., Green, J.R. and Bilbe, G. (1993) *J. Biol. Chem.* 268, 7075–7080.
- [26] Bernasconi, P., Rausch, T., Struve, I., Morgan, L. and Taiz, L. (1990) *J. Biol. Chem.* 265, 17428–17431.
- [27] Graf, R., Novak, F.J., Harvey, W.R. and Wicczorek, H. (1992) *FEBS Lett.* 300, 119–122.
- [28] Yi, Y. and Samuelson, J. (1994) *Mol. Biochem. Parasitol.* 66, 165–169.
- [29] Karcz, S.R., Herrmann, V.R. and Cowman, A.F. (1993) *Mol. Biochem. Parasitol.* 58, 333–344.
- [30] Karcz, S.R., Herrmann, V.R. and Cowman, A.F. (1994) *Mol. Biochem. Parasitol.* 65, 123–133.